# Calcium pyrophosphate dihydrate crystal deposition in the ligamentum flavum of the cervical spine: histopathological and immunohistochemical findings

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# Abstract Object

To investigate the histopathological and immunohistochemical properties of degenerative changes in the ligamentum flavum of the cervical spine with calcium crystal deposition.

# Methods

Sections of the calcified ligamentum flavum harvested from 26 patients who required cervical decompression were examined by scanning electron microscopy (SEM), energy dispersive X-ray microanalysis, immunohistochemical staining [for transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF), Sox9, and Msx2] and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labelling (TUNEL) method (for cell apoptosis).

# Results

Energy dispersive x-ray microanalysis and SEM confirmed the deposited calcium to be calcium pyrophosphate dihydrate (CPPD) crystals. The calcified ligamentum flavum showed disorganisation of the elastic fibre bundles together with increased collagen fibrils in the matrix. Abundant hypertrophic chondrocytes were noted around the calcified lesions, which were strongly immunoreactive to TGF-β and VEGF. Staining for Sox9 was positive in metaplastic chondrocytes but negative in hypertrophic chondrocytes. Both chondrocytes and mesenchymal cells were positive for Msx2. TUNEL-positive hypertrophic chondrocytes were significantly more noticeable in nodular than diffusely scattered type of CPPD deposition.

# Conclusions

Calcium crystal deposition in the cervical ligamentum flavum seems to progress with reduction in elastic fibres, increase in collagen fibrils in the matrix, and migration of metaplastic hypertrophic chondrocytes, whose differentiation is controlled by cytokines and transcriptional factors, and potentially regulate crystal formation. The presence of abundant TUNEL-positive hypertrophic chondrocytes around CPPD deposition suggests that materials from apoptotic cells play some role in crystal deposition.

# Key words

Cervical spine, ligamentum flavum, degeneration, calcium pyrophosphate dihydrate crystal, immunohistochemistry.

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#### Introduction

Calcium pyrophosphate dihydrate (CPPD) crystal deposition in the cervical ligamentum flavum is associated with insidious progression of neurological compromise (1-4). This pathologic condition may occur as part of systemic chondrocalcinosis or in isolation (5), but it is considered to be frequently present in association with generalised connective tissue degeneration. The spinal canal compromise associated with slow and progressive CPPD crystal deposition in the ligamentum flavum is primarily due to the mass effect of such deposition. The cervical ligamentum flavum is only about 1-2 mm thick, and thus a small nodular lesion with ligamentous thickening results mechanically in dorsal cord compression especially with the "buckling effect" on the cord during neck extension position, ultimately resulting in the development of myelopathy (6).

Proliferation of degenerated collagen fibrils, stimulation by increased proteoglycan levels, and hypertrophic chondrocytes that secrete amorphous material as well as proteoglycans, are typical findings of calcified ligamentous matrix (7, 8). Furthermore, degeneration of the elastic fibres and increased number of collagen fibrils are also sometimes observed on histopathological examination of calcified ligamentum flavum (9-11). Quantitative analyses of the expanding chondrometaplastic areas, regularity of arrangement of elastic fibre bundles, and intraligamentous content associated with degeneration of ligaments have also been reported. Our group reported previously the role of proteases, such as elastase and chymotrypsin, in the degeneration of elastic fibres in the lumbar ligamentum flavum, and that estrogens as well as transforming growth factor (TGF)-ß regulated crystal formations in chondrocytes (12-14). Based on these findings, we speculated that the degeneration process associated with small blood vessel formation, the expression of certain cytokines, and the presence of metaplastic chondrocytes could contribute to the deposition of CPPD crystals.

Since the anatomy and histology of the cervical ligamentum flavum are

different from those of the lumbar spine, we decided to investigate the cervical calcified ligamentum flavum. In the present study, we investigated the histopathological and immunohistochemical properties of CPPD crystal deposition in the cervical spine, with reference to our previous findings in the lumbar spine (6).

### Material and methods

Between 1994 and 2008, 465 Japanese patients underwent posterior decompressive surgery for proven cervical myelopathy. Among them, 26 patients (11 men; 15 women; mean age 73.1 years, range 50-86; mean follow-up period, 2.1 years) had distinctive clinical and radiological evidences of spinal cord compression vis a tergo by CPPD crystal deposition. None of the patients showed evidence of genetically-related bone, joint or musculoligamentous tissue abnormalities, or had taken corticosteroids, immunodepressants, bisphosphonate, ethidronate sodium and/ or tumour necrosis factor- $\alpha$  antagonist. Blood tests did not routinely include investigation of vitamin D, parathyroid hormone and calcitonin, serum bonespecific alkaline phosphatase or urinary N-telopeptide for type I collagen, but all patients were negative for rheumatoid factor. In all patients, radiographs excluded the presence of ankylosing spondylitis, diffuse idiopathic skeletal hyperostosis, and Paget's disease. In a previous study (6) that reviewed 66 cases with cervical ligamentum flavum CPPD crystal deposition, the prevalence of associated calcification in the knee meniscus was 38%, intervertebral disc 23%, posterior longitudinal ligament 11%, hip joint 11%, pubic symphysis 11%, shoulder joint 8%, peri-odontoid area (crowned dens syndrome) 6%, and wrist joint (triangular ligament) 5%. However, in the current series, such systemic radiographic survey was not performed mainly due to medical costs and ethical reasons of increased radiation exposure.

Neurological examination was performed on admission and at follow-up. The calcified lesion was assessed at each spinal level on radiographs and/or highresolution CTs, and its morphology was grossly categorised as nodular or diffuse type, as described previously by our group (2, 6). The lesion was visualised as a vague radiolucent mass between two laminae on sagittal radiographs, and as a clearly margined nodular lesion or vague spotty calcification beneath the lamina. On high-resolution MRIs (1.5 Tesla Signa, Milwaukee, WI), the calcified lesion appeared as a low-signal intensity lesion, with clear evidence of compression of the spinal cord vis a tergo.

Surgical treatments included osteoplastic en bloc open-door laminoplasty for 23 patients with multi-segmental compressive lesions anteriorly and/or posteriorly, and microscopic laminotomy for three sustaining single level lesion posteriorly. The postoperative neurological outcome as assessed by the criteria of Odom et al. (15) was classified as excellent (every preoperative symptom relieved and abnormal findings improved), good (minimal persistence of preoperative symptoms and abnormal findings unchanged or improved), fair (definite relief of some preoperative symptoms, but other symptoms unchanged or slightly improved), or poor (symptoms and signs unchanged or worse). Surgeries were performed by a single senior surgeon (HB) who did not participate in histological and immunohistochemical assessments. The principal authors (ESM, TY), and two histopathologists (KU, SK) who were blinded to the surgical findings, were responsible for all histopathological and crystallographic investigations. All other data were further assessed by other independent observers (HN, RS). The Human Ethics Review Committee

of our University approved the current study protocol and a signed consent form was obtained from each subject.

## Histopathological and immunohistochemical examinations and terminal deoxynucleotidyl transferase (TdT)mediated dUTP-biotin nick-end labelling (TUNEL) staining

Surgically obtained *en bloc* ligamentum flavum tissues were fixed with 10% buffered formaldehyde for 48 hours at 4°C and then embedded with paraffin. Serial 4-µm thick sections were examined after deparaffinization with xylene and replaced by 99.5% ethanol, followed by haematoxylin and eosin and elastica van Gieson staining.

For immunohistochemical staining, serial 4 µm-thick sections were prepared from the paraffin embedded specimens, and then deparaffinized with xylene followed by 99.5% ethanol. After washing with water, the intrinsic peroxidase was blocked with 0.3% H<sub>2</sub>O<sub>2</sub>-methanol solution at 20°C for 10 minutes and washed with phosphate buffered saline (PBS; pH 7.4). In the next step, the sections were reacted with BLOCKING agent [PBS containing carrier protein and 15 mM sodium azide (LSAB kit, Lot 00075, Dako, Glostrup, Denmark)] at 20°C for 10 minutes. This was followed by reaction overnight with the following primary antibodies at 4°C: mouse monoclonal anti-TGF-β (MAB1835, Lot CCI02, R&D Systems, 500 µg dissolved in 1ml PBS), mouse monoclonal anti-vascular endothelial growth factor (VEGF) (SC7269, Lot G231T, Santa Cruz Biotechnology, 200 µg IgG dissolved in 1 ml PBS with 0.1% sodium azide and 0.2% gelatin), rabbit polyclonal anti-Sox9 (sc-20095, Lot DO406, Santa Cruz Biotechnology, containing 200 µg IgG in 1 ml PBS with <0.1% sodium azide and 0.1%gelatin), and rabbit polyclonal anti-Msx-2 (sc-15396, Lot CO404, Santa Cruz Biotechnology, containing 200 µg IgG in 1 ml PBS with <0.1% sodium azide and 0.1% gelatin). Sections were further reacted with goat anti-mouse immunoglobulin antibodies conjugated to peroxidase labelled-dextran polymer (EnVision<sup>™</sup>, peroxidase, Dako Corp., Carpentaria, CA) for monoclonal antibodies and with goat anti-rabbit immunoglobulin antibodies (EnVision+, peroxidase, Dako) for polyclonal antibodies at 20°C for 45 minutes and rinsed with PBS at pH 7.4 (16). In order to visualise peroxidase colour reaction, the sections were incubated with 3, 3-diaminobenzidine (DAB)-HCl solution (CB090; Dojin Chemicals, Tokyo, Japan, 50 mg dissolved in 100 ml of 0.05 M TRIS-HCl buffer, pH 7.4) at 20°C for 10 minutes, and washed in water. Nuclear counterstaining was carried out with haematoxylin. Specimens stained by DAB were mounted with Biolite (Oken, Tokyo) and examined under a light microscope.

Apoptotic cell death was assessed by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine 5triphosphate (d-UTP) nick-end labelling (TUNEL) technique. The specimens were deparaffinized and dehydrated according to standard protocols, the tissue sections (4 µm) were incubated with BLOCKING solution (0.3% H<sub>2</sub>O<sub>2</sub> in methanol) for 30 minutes at room temperature. After rinsing with PBS (pH 7.2), the sections, were incubated for 15 minutes at 37°C with proteinase K solution (10 µg/ml in 10 mM Tris-HCl buffer, pH 7.4) and rinsed twice with PBS. TUNEL reaction mixture [50 µl enzyme solution (TdT from calf thymus in storage buffer) added to 450 µl label solution (nucleotide mixture in reaction buffer) and mixed well to equilibrate components] was prepared immediately before use, placed on slides (50 µl/ slide), and incubated for 60 minutes at 37°C. For the negative control, label solution without terminal transferase was placed on slides (50 µl/slide) instead of the TUNEL reaction mixture. These were rinsed three times with PBS, added to DAB substrate (10  $\mu$ l 30% H<sub>2</sub>O<sub>2</sub> added to 5 mg /ml DAB in 50 mM Tris-HCl buffer, pH 7.4) and incubated for 10 minutes at room temperature. The slides were rinsed with distilled water and counterstained with methyl green (1%) for 5 minutes. After mounting, the specimens were analysed under light microscopy.

## Scanning electron microscopy and x-ray microanalysis

Sections obtained from each specimen were examined by scanning electron microscopy (SEM) and some sections were examined by energy dispersive x-ray microanalysis. Sections were fixed with 2.5% glutaraldehyde (Wako Pure Chemicals, Osaka, Japan) water solution at 4°C for 2 hours, and then immersed in 1% osmium tetroxide (Merck, Darmstadt, Germany) at 4°C for 2 hours. After dehydration with serial concentrations of ethanol followed by absolute isoamyl acetate solution (Nakalai, Kyoto, Japan), the specimen

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1       1       C45.56       Noth       ·:       ·:       C45.56       Noth       ·:  <	Case	Age (yr), Sex	Associated diseases	Levels of calcium deposition	Type of deposition	Other spinal diseases	Pain U/L	Numbness U/L	Motor Def. U/L	Sensory Def. U/L	Gait instability	Bladder dysfunction	Abnormal reflex	Arm radicular sign	Treatment	Result	Follow up (yr)
3       9.M       ·       C34453.647       Dilus       ··       ·       Lumunglus       Pin       ··       Lumunglus       Pin       ··       C44453.647       Dilus       ··       ·       Lumunglus       Pin       ··       C44453.647       Dilus       ··       ·	-	81, F	,	C4-5, 5-6	Nodular		- / -	+/+	+/+	+/+	+	ı	+	ı	Laminoplasty	excellent	0.3
3       0, F	5	79, M	,	C3-4, 4-5, 5-6, 6-7	Diffuse		+ / -	+/+	+/+	+/+	+	+	+	ı	Laminoplasty	fair	1.3
4       6.M.       Myoniporitiem       C3.3.4.       Diffe       ····       ···       ···       ···       ····       ···       ····       ····       ····       ····       ····       ····       ····       ····       ····       ····   ····       ···	3	67, F		C3-4, 4-5, 5-6, 6-7	Diffuse	C3-6 OPLL	- / +	- / +	+/+	- / +	+	ı	ı	+	Laminoplasty	poor	5.3
5       7.5.1       DM       C:3-4       Diffication       D	4	65, M I:	hypothyroidism	C2-3, 3-4	Diffuse		- / -	+/+	- / +	- / +	+	I	+	+	Laminoplasty	excellent	2.1
6       73.1       1.       C. 4.4.5       Modular       · <	5	75, F	DM	C3-4	Diffuse		- / -	+/+	- / +	+/+	ı	+	+	ı	Laminoplasty	excellent	9.3
$ \begin{array}{ ccccccccccccccccccccccccccccccccccc$	9	73, F		C3-4, 4-5	Nodular		- / +	+/+	+/+	+/+	+	+	+	+	Laminoplasty	good	2.3
8 7 0.0 1 c (-1) (-2) (-2) (-2) (-2) (-2) (-2) (-2) (-2	7	75, M	DM	C3-4, 4-5, 5-6, 6-7	Diffuse	C2-5 OPLL	- / +	+/+	+/+	+/+	+	,	+	,	Laminoplasty	good	0.3
9       76, F       .       C34,4-5       Diffus       ·   ·   ·   ·       · <td>8</td> <td>50, M</td> <td></td> <td>C4-5, C5-6</td> <td>Diffuse</td> <td></td> <td>- / +</td> <td>- / +</td> <td>- / +</td> <td>- / -</td> <td>ı</td> <td>ı</td> <td>·</td> <td>+</td> <td>Laminoplasty</td> <td>excellent</td> <td>0.5</td>	8	50, M		C4-5, C5-6	Diffuse		- / +	- / +	- / +	- / -	ı	ı	·	+	Laminoplasty	excellent	0.5
10       79,M        C34,4,5,7:11       Diffus        +       +       +       +       +       +       +       Main operation       good       30         11       8,4        C34,4,5,7:11       Diffus	6	76, F		C3-4, 4-5	Diffuse		- / -	+/+	+/+	+/+	+	ı	+	·	Laminoplasty	good	9.6
	10	79, M	,	C3-4, 4-5, C7-T1	Diffuse		- / +	+ / +	- / +	+/+	+	+	+	+	Laminoplasty	good	3.0
1     8.5.F      C3.4     Diftus      1     <	11	84, F	,	C3-4	Diffuse		- / -	+/+	+/+	+/+	+	+	+	ı	Laminoplasty	fair	0.3
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	15	76, F	,	C4-5, 5-6	Diffuse		- / +	- / +	- / +	+/+	+	I	+	ı	Laminoplasty	good	0.2
17 $39, M$ DM $C56, 677$ NodularCDH $-1/2$ $+1/4$ <td>16</td> <td>74, F</td> <td></td> <td>C6-7</td> <td>Nodular</td> <td></td> <td>- / -</td> <td>+/+</td> <td>- / -</td> <td>+/+</td> <td>+</td> <td>,</td> <td>+</td> <td>,</td> <td>Laminoplasty</td> <td>excellent</td> <td>0.3</td>	16	74, F		C6-7	Nodular		- / -	+/+	- / -	+/+	+	,	+	,	Laminoplasty	excellent	0.3
1871,M-C45,5.6, C7-T1Diffuse- $+/ +/ +/ +/ +/ +/ +/ +/ +/ +/ +/ +/ +/ -/-$	17	59, M	DM	C5-6, 6-7	Nodular	СDH	- / -	+ / +	+/+	+/+	+	+	+	+	Laminotomy, C5-6 ADIF	good	0.2
1979, MDM $C34,4.5$ Diffuse- $+/$ <th< td=""><td>18</td><td>71, M</td><td>,</td><td>C4-5, 5-6, C7-T1</td><td>Diffuse</td><td></td><td>- / +</td><td>- / +</td><td>- / +</td><td>- / +</td><td>ı</td><td>ı</td><td>+</td><td>+</td><td>Laminoplasty</td><td>good</td><td>3.0</td></th<>	18	71, M	,	C4-5, 5-6, C7-T1	Diffuse		- / +	- / +	- / +	- / +	ı	ı	+	+	Laminoplasty	good	3.0
20 8. M $-$ C4-5 Nodular $ -/ +/+$ $+/+$ $+/+$ $+/+$ $+/+$ $+/+$ $+/+$ $-/-$ Laminoplary fair 0.9 21 70. F $-$ C5-6 Nodular $ +/+$ $+/+$ $-/ +/+$ $+/+$ $-/ -$ Laminoplary good 0.8 22 8. M DM C4-5 Diffuse CDH $+/ +/+$ $+/+$ $-/ +/+$ $+$ $+$ $+$ $+$ $+$ $-$ Laminoplary good 0.3 23 59. F DM C5-6 Nodular $ -/+$ $+/+$ $+/+$ $-/ +/+$ $+$ $+$ $+$ $+$ $+$ $-$ Laminoplary good 0.3 24 78. M C4-5, C5-6, C6-7 Diffuse C3-5 OPLL $+/ +/+$ $+/ +/+$ $+/+$ $+$ $+$ $+$ $+$ $-$ Laminoplary good 0.2 25 $77.$ F DM C4-5, C5-6, C6-7 Diffuse C3-5 OPLL $+/ +/+$ $+/ +/+$ $+/+$ $+$ $+$ $ -$ Laminoplary good 0.2 26 $70.$ F DM C4-5, C5-6, C6-7 Diffuse C3-5 OPLL $+/ +/+$ $+/ +/+$ $+$ $+$ $+$ $ -$ Laminoplary good 0.2 27 $7.$ F DM C4-5, C5-6, C6-7 Diffuse C3-5 OPLL $+/ +/+$ $+/ +/+$ $+$ $+$ $+$ $+$ $  -$ Laminoplary good 0.2 28 $77.$ F DM C4-5, C5-6, C6-7 Diffuse C3-5 OPLL $+/ +/+$ $+/ -/ +/+$ $+$ $+$ $+$ $+$ $         -$	19	79, M	DM	C3-4, 4-5	Diffuse		- / +	+ / +	+/+	+/+	+	+	+	+	Laminoplasty	fair	1.6
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	20	86, M		C4-5	Nodular	ı	- / -	+/+	+/+	+/+	+	+	+	ı	Laminoplasty	fair	0.9
22 $S_{0}$ , M DM $C_{4}$ . DM $C_{5}$ . Diffuse CDH $+/ +/+$ $+/+$ $-/ +/+$ $+/+$ $+/-$ Laminotomy, good 0.3 23 $S_{0}$ , F DM $C_{5}$ . Good Nodular $ -/+$ $+/+$ $+/ +/+$ $+/ +/+$ $+/ +/+$ $+/ -/-$ Laminotomy excellent 0.3 24 $7_{0}$ , M $C_{4}$ . S, C, Go-7 Diffuse C.3.5 OPLL $+/ +/+$ $+/ +/+$ $+/ +/+$ $+/ +/-$ Laminoplasty good 0.2 25 $7_{7}$ , F DM $C_{4}$ . S, C, S-6, Nodular $-/ -/ +/+$ $+/ +/+$ $+/ +/+$ $+/ -/-$ Laminoplasty good 0.2 26 $7_{6}$ , F $-$ C.3.4 Diffuse C.3.5 OPLL $+/ +/+$ $+/ +/+$ $+/ +/+$ $+/ -/-$ Laminoplasty good 0.2 26 $7_{6}$ , F $-$ C.3.4 Diffuse C.3.5 OPLL $-/ +/+$ $+/ +/+$ $+/ -/ +/ +/ -/-$ Laminoplasty good 0.2 26 $7_{6}$ , F $-$ C.3.4 Diffuse C.3.5 OPLL $-/ -/ +/+$ $+/ -/ +/ +/ +/ -/ -/ -/ -/ -/ -/ -/ -/ -/ -/ -/$ $-/$ $-/$ $-/$ $-/$ $-/$ $-/$ $-/$ $-/$ $-/$ $-/$ $-/$	21	70, F		C5-6	Nodular	ı	+/+	+/+	+ / -	+/+	ı	ı	+	ı	Laminoplasty	good	0.8
2359, FDMC5-6Nodular/++/+-/-+/++/++/0.22478, MC4-5, C5-6, C6-7DiffuseC3-5 OPLL+/-+/++/+++++12577, FDMC4-5, C5-6, C6-7DiffuseC3-5 OPLL+/-+/++/+++++12676, FC3-4Diffuse-/-+/++/-+/++/+++10.22676, FC3-4Diffuse-/-+/++/-+/++/+++11OPLL: cosification of posterior longitudinal ligament; CDH: cervical disc hemiation; ADIF: anterior decompression and interbody fusion; subtotal: subtotal spondylectomy; U/L: upper extremities; Def: deficit; DM: Diabetesnellitus.	22	58, M	DM	C4-5	Diffuse	СDH	- / +	+ / +	- / -	+ / -	+	+	+	ı	Laminotomy, C4-5 ADIF	poog	0.3
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	23	59, F	DM	C5-6	Nodular		+ / -	+/+	- / -	+/+	+	,		,	Laminotomy	excellent	0.3
25 77, F DM C4-5, C5-6 Nodular $-/ +/+$ $-/ +/+$ $-/-$ Laminoplasty excellent 0.2 26 76, F C3-4 Diffuse $-/ +/+$ $+/ +/+$ $+/ +/+$ $+$ Laminoplasty excellent 0.2 OPLL: ossification of posterior longitudinal ligament; CDH: cervical disc hermiation; ADIF: anterior decompression and interbody fusion; subtotal: subtotal: subtotal spondylectomy; U/L: upper extremities; Def: deficit; DM: Diabetes mellitus.	24	78, M		C4-5, C5-6, C6-7	Diffuse	C3-5 OPLL	- / +	+/+	- / +	+/+	+	+	+	+	Laminoplasty	good	0.2
26 76, F C.3-4 Diffuse -/- +/+ +/- +/+ - +/- +/- Eminoplasty good 0.2 OPLL: cossification of posterior longitudinal ligament; CDH: cervical disc hemiation; ADIF: anterior decompression and interbody fusion; subtotal: subtotal spondylectomy; U/L: upper extremities; Def: deficit; DM: Diabetes mellitus.	25	77 , F	DM	C4-5, C5-6	Nodular		- / -	+ / +	- / -	+/+	+	I	+	ı	Laminoplasty	excellent	0.2
OPLL: ossification of posterior longitudinal ligament; CDH: cervical disc herniation; ADIF: anterior decompression and interbody fusion; subtotal: subtotal: subtotal spondylectomy; U/L: upper extremities / lower extremities; Def: deficit; DM: Diabetes mellitus.	26	76, F		C3-4	Diffuse		- / -	+/+	- / +	+/+	+	I	+	+	Laminoplasty	good	0.2
	OPLL: mellitu	ossification s.	of posterior long	gitudinal ligament; CDF	H: cervical disc	herniation; ADIF.	anterior c	lecompression .	and interbody	fusion; subtc	otal: subtotal	spondylecton	ıy; U/L: upp	er extremities /	lower extremities;	Def: deficit; D	M: Diabetes

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was allowed to dry in a critical point dryer (HCP-2; Hitachi, Tokyo), and then coated with gold-palladium (Eiko Engineering, Ibaraki, Japan) by evaporation using an ion coater (IB-3; Eiko Engineering). In the final stage, SEM and an energy dispersive x-ray microanalyser (model S-3400N; Hitachi) were used to identify calcium crystals.

### Results

#### Clinical findings

Table I summarises the clinical demographic data, radiological findings, and surgical outcome of participating patients. Eight patients had long-standing diabetes mellitus and one had hypothyroidism. Symptoms and signs of myelopathy were evident in 16 patients, myeloradiculopathy in seven, and radiculopathy in three. After surgery, 20 patients attained excellent or good outcome, but one (Case 3) with a sustained concomitant ossified posterior longitudinal ligament showed poor results. None of the patients had symptomatic recurrence of CPPD deposition at the surgical sites or other sites during the follow-up.

Ligamentum flavum calcification was observed at C2-3 level in one, C3-4 level in 13, C4-5 level in 17, C5-6 level in 12, C6-7 level in five, and C7-T1 in three. Ten patients showed nodular calcification (Fig. 1), while 16 exhibited diffuse type of deposition (Fig. 2). No significant features were noted in the cervical spine in terms of osseous anatomy, suggestive of either nodular or diffuse type calcium deposition.

# Scanning electron microscopy and histological findings

The deposited calcium crystals varied morphologically, appearing as pin-like, rod-like, or rectangular crystals when observed under SEM (Fig. 3a, 3b). The diameter of the crystals was approximately 4  $\mu$ m or slightly larger. X-ray microanalysis indicated the crystal was consistent with CPPD (Fig. 3c, 3d). Macroscopic examination of the calcified ligamentum flavum showed granulated white chalky mass, and the lesion was often observed primarily in the dorsal side of the ligament with grad-

ual extension towards the dural side.



**Fig. 1.** Imaging findings and gross appearance of the nodular type calcium pyrophosphate dihydrate (CPPD) crystal deposition (Case 23, Table I). The nodular type lesion at C5-C6 level exhibits spinal cord compression *vis a tergo*, causing intramedullary hyperintensity signal at the corresponding level (**a**, **b**, T1-weighted MRIs; **c**, **d**, T2-weighted MRIs). On high-resolution CT image (**e**), the nodular lesions (white arrows) can be seen beneath the lamina with clear margins. Gross appearance of the resected ligamentum flavum at C5-C6 showing white, chalky, granular calcium deposition (black arrows) primarily in the deep layer (dorsal side) of the ligament (**f**).



**Fig. 2.** MRI and high-resolution CT findings, and gross appearance of the diffuse type of CPPD deposition (Case 22, Table I). The calcified ligamentous lesion at C4-C5 level is associated with a significant compression of the spinal cord posteriorly ( $\mathbf{a}$ ,  $\mathbf{b}$ , T1-weighted MRIs;  $\mathbf{c}$ ,  $\mathbf{d}$ , T2-weighted MRIs). On MRIs, the calcium-containing ligament exhibits isointensity ( $\mathbf{a}$ ,  $\mathbf{b}$ ) and hyperintensity ( $\mathbf{c}$ ,  $\mathbf{d}$ ) signals. On high-resolution CT image ( $\mathbf{e}$ ), diffusely scattered calcified lesions (white arrows) close to the lamina are visualised. Note the white and chalky gross appearance of the ligamentum flavum at C4-C5 level, and diffuse deposition of calcium (black arrows) in the cephalad attachment of the deep layer (dorsal side) of the ligament ( $\mathbf{f}$ ).

Microscopic examination showed irregular arrangement of the elastic fibres, abnormally small diameter of fragmented elastic fibres, and thick bundles of collagen fibres. The above gross findings were observed in both the nodular (Fig. 4a, 4b) and diffuse type (Fig. 4c, 4d) of calcium deposition. In both types of depositions, wide amorphous fibrotic areas with reduced number of elastic fibres and increased collagenous tissue were frequently evident. Different from the nodular type of deposition, calcium deposition in the diffuse type was scattered among the matrix that showed ruptured elastic fibre bundles. A number of chondrocytes were found around the calcium deposition-containing matrix, together with proliferation of small blood vessels in the periphery of calcified lesion.

## Immunohistochemical findings

Strong immunostaining for TGF- $\beta$  was noted in hypertrophic chondrocytes with chondrometaplasia in the matrix of the ligamentum flavum. A number of TGF- $\beta$ -positive hypertrophic chondrocytes were found around calcium crystal deposits in both nodular and diffuse types of depositions (Fig. 5a).

Hypertrophic chondrocytes in the matrix of the degenerated ligamentum flavum were immunostained for VEGF. A large number of VEGF-positive hypertrophic chondrocytes were seen around the calcium crystals in both types of depositions (Fig. 5b).

The chondrocytes in the degenerated ligaments were also immunopositive for Sox9 (Fig. 5c). However, the immunopositivity was limited to the seemingly pre-mature chondrocytes; the hypertrophic chondrocytes in close vicinity of calcium deposits were negative for Sox9. Furthermore, immunostaining for Sox9 was also noted in mesenchymal cells present around the calcified lesion. These mesenchymal cells were found exclusively in the degenerative fibres around the calcium deposits.

Immunostaining for Msx2 was significant in the hypertrophic chondrocytes in ligaments with calcium crystal deposits (Fig. 5d). In particular, Msx2 showed significant immunoreactivity in hypertrophic chondrocytes present in the matrix of degenerated ligament around the calcified lesions. Furthermore, marked Msx2 immunopositivity was also evident in mesenchymal cells.

Some of the hypertrophic chondrocytes in the degenerated matrix were also TUNEL-positive. The nuclei of these chondrocytes showed pigmentation



**Fig. 3.** Scanning electron micrographs of the lesion showed calcium crystals of various sizes and shapes ( $\mathbf{a}$ ; x1500;  $\mathbf{b}$ ; x7500). The measured point indicated by lunate circle in  $\mathbf{c}$  and x-ray microanalysis ( $\mathbf{d}$ ) confirmed the minerals to be CPPD crystals.



**Fig. 4.** Photomicrographs showed the presence of chondrocytes around calcium deposits and irregular arrangement of the elastic fibres. In nodular type deposition, the area of calcium deposits was oval or semi-oval in shape with a clear margin from the matrix (**a**; haematoxylin and eosin staining, **b**; elastica van Gieson staining; each magnification, x10). In diffuse type deposition, amorphous deposition areas were scattered within the ligamentous matrix (**c**; haematoxylin and eosin staining, **d**; elastica van Gieson staining; each magnification, x10). **Ca**: calcium deposition.

and cohesion, suggestive of cell apoptosis. In particular, these TUNEL-positive chondrocytes were mostly seen in areas close to the calcium deposits in cases of nodular type (Fig. 6a, 6b) compared with the diffuse calcification (Fig. 6c, 6d).

### Discussion

Calcium deposition in the cervical ligamentum flavum increases its thickness together with loss of elasticity and may eventually change to a hard mass lesion with a significant impingement of the spinal cord posteriorly or postero-



**Fig. 5.** Immunohistochemical findings of the calcified lesion. Hypertrophic chondrocytes positive for TGF- $\beta$  (**a**) and VEGF (**b**) were abundant around the area of calcium deposition. Chondrocytes were immunopositive for Sox9 (**c**) but immunonegative for hypertrophic chondrocyte. Msx2 (**d**) showed marked immunoreactivity in chondrocytes and mesenchymal cells present in the area of severely degenerated matrix around the calcified lesion (**a**-**d**: magnification, ×20). Negative control (nc) photographs are shown on the right side of each figure.



**Fig. 6.** Photomicrographs of calcified tissues stained by terminal deoxynucleotidyl transferase (TdT)mediated deoxyuridine 5-triphosphate (d-UTP) nick end-labelling (TUNEL). In nodular type deposition, TUNEL-positive chondrocytes were present around the crystal deposition (**a**, haematoxylin and eosin staining; **b**, TUNEL staining). In diffuse type deposition, TUNEL-positive chondrocytes were scattered widely in the matrix (**c**, haematoxylin and eosin staining; **d**, TUNEL staining). **a-d**: magnification, x20. **Ca**: calcium deposition.

laterally. Several studies (17-19) have discussed the clinical significance of calcium deposition in this area. While the exact prevalence of CPPD crystal deposition is unknown, the incidence probably increases with age and is estimated to be 10-25% in elderly people aged  $\geq$ 75 years (3, 12-14, 20). The condition is more prevalent in females and patients with diabetes mellitus, atherosclerosis and hypertension are considered to be at higher risk than others. Clinical presentations include sensory dysfunction affecting the extremities,

power loss, reflex abnormalities, gait instability, and deterioration of bladder function in advanced cases. A review of 60 reported cases (3, 6, 10, 17, 21-23) with cervical myelopathy caused by CPPD crystal deposition provides some important clinical information. Firstly, initial neurological signs and symptoms include prominent coordination deficit or spinal ataxia (positive Romberg's sign) and loss of tactile sensation resulting from dorsal cord compression, either continuously or intermittently. Secondly, due to posterior compression of the nerve root(s), radicular pain and motor deficit are rather less significant than in patients with intervertebral disc herniation. Radiological findings of CPPD crystal deposition in the mid-cervical spine include vague calcified area in the interlaminar space on lateral radiographs, oval or semioval and/or diffusely scattered calcified lesions adjacent to the inner cortices of the laminae on CTs, and lowsignal intensity lesions in the areas of the ligamentum flavum on sagittal and transaxial T1-weighted images of highresolution MRIs. On MRIs, the lesion often shows no rim enhancement after gadolinium administration, and the posterior "buckling effect" of the ligamentum flavum is more significant on MR images in neck-extended position. We recommend obtaining a detailed radiological work-up such as lateral radiographs and CTs; nevertheless, the first choice of radiological examination should be high-resolution MRIs.

Histopathologically, previous reports (6, 12-14) described significant degeneration and loss of elastic fibre bundles in the calcified ligamentum flavum, together with appearance of some metaplastic chondroblast-like mesenchymal cells, as well as apoptosis of fibroblasts with increased collagen fibrils and fibrosis around the CPPD crystal deposition. Invasion of small blood vessels around the CPPD deposition was significant in the superficial layer of the ligament rather than the deep one, but in focal (nodular) deposition, this neovascularisation was widespread in the latter layer. The deposits of crystal were mainly well delineated and finely granular, but in some cases they were

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scattered diffusely without any significant inflammatory reactions. In the current series, we can summarise the histopathological findings to marked degenerative changes (thinning and fragmentation) of the elastic fibres with some neovascularisation and infiltration of mesenchymal fibroblast-like and chondroblast-like cells.

Immunohistochemically, a number of hypertrophic chondrocytes and fibroblast-like mesenchymal cells that infiltrated around the calcified deposits were significantly positive for basic fibroblast growth factor and TGF-β. In our previous studies (12-14), we reported that basic fibroblast growth factor, possibly secreted from these cells and regulated by TGF- $\beta$ , could promote neovascularisation in the matrix where metaplastic chondrocytes or chondroblast-like cells migrate. Similar to the findings of Yahia et al. (9), we observed marked chondrometaplasia in the calcified ligamentum flavum and metaplastic hypertrophic chondrocytes positive for S-100 protein and containing elastase as well as chymotrypsin. S-100 protein is known as a specific binding peptide to calcium crystal. We speculate that the newly appeared mesenchymal fibroblast-like cells in the degenerated areas of elastic fibre bundles were transformed via the TGF- $\beta$  and other unknown cytokines, to S-100protein-postive chondrocytes, which contributed to crystal deposition in certain areas of the degenerated ligament. The results suggest that the hypertrophic chondrocytes could play a role in crystal formation, since they were abundant in the degenerated ligaments accompanied by metaplastic chondroid matrix. Yahia et al. (9) considered the metaplastic chondrocytes to be derived from fibroblasts. We found hypertrophic and seemingly premature chondrocytes in areas with ruptured elastic fibres containing a number of mesenchymal cells or fibroblast-like cells. Sox9 is a member of Sox family of transcription factors, which is expressed in chondroprogenitor cells and chondrocytes, while its expression is significantly decreased in hypertrophic chondrocytes (24). In contrast, Msx2 prevents ligament fibroblasts from undergoing osteoblastic cells or

chondrocyte differentiation (25). These promoters might regulate the process of chondrogenesis and chondrocyte differentiation from mesenchymal cells or fibroblast-like cells to mature chondrocytes. The hypertrophic chondrocytes in the focally degenerated ligament, however, were significantly TUNELpositive, especially around crystal deposition. It is not clear whether the chemical change in matrix induced apoptosis of hypertrophic chondrocytes or these cells themselves underwent programmed cell death triggered by biochemical alteration of chondroid matrix secondary to ligamentous degeneration, or whether apoptosis was induced by the crystals and/or matrix vesicles (13). However, it seems that TUNEL-positive hypertrophic chondrocytes correlate with CPPD crystal deposition in the degenerated ligament flavum.

In summary, CPPD crystal deposits in the ligamentum flavum in the cervical spine showed nodular or diffuse pattern. Histopathological examination showed reduction in elastic fibres associated with appearance of mesenchymal fibroblasts and fibroblast-like cells as well as neovascularisation in both types of calcium deposits. We found a number of metaplastic hypertrophic chondrocytes with cluster formation around crystal deposition sites, and these cells were TUNEL-positive. These processes seem to be controlled by cytokines and/or transcriptional factors, and we speculate that metaplastic hypertrophic chondrocytes play a critical role in CPPD crystal deposition in the cervical ligamentum flavum.

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